Design and construction of a new *Drosophila* species, *D.synthetica*, by synthetic regulatory evolution

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Summary

Synthetic biology is an area of biological research that combines science and engineering[1,2]. Synthetic creations can have practical applications and/or help us understand the complexity of natural systems[1,2]. Independently, regulatory evolution has shown that biological novelty can arise from the rearrangement of preexisting elements into new regulatory networks[3-11]. Here, I merge the principles of synthetic biology[1,2] and regulatory evolution[3-11] to create a new species[12-15] with a minimal set of known elements. Using preexisting transgenes and recessive mutations of *Drosophila melanogaster*, a transgenic population arises with small eyes and a different venation pattern that fulfills the criteria of a new species according to Mayr's "Biological Species Concept"[7,10]. The genetic circuit entails the loss of a non-essential transcription factor and the introduction of cryptic enhancers. Subsequent activation of those enhancers causes hybrid lethality. The transition from "transgenic organisms" towards "synthetic species", such as *Drosophila synthetica*, constitutes a safety mechanism to avoid hybridization with wild type populations and preserve natural biodiversity[16-18]. *Drosophila synthetica* is the first transgenic organism that cannot hybridize with the original wild

type population but remains fertile when crossed with other transgenic animals.

Highlights:

- A new species is created by design in the genus *Drosophila*.
- The population, with small eyes and different venation, conforms to the most stringent definitions of species.
- "Synthetic species" can have practical applications.
- "Synthetic regulatory evolution" can be a general mechanism to create "synthetic species".

Species are the basic units of biological classification and the question of how new species arise is fundamental for understanding evolution[7-11]. Previous work in several species of *Drosophila* produced fundamental contributions regarding the genetics of speciation[7-10]. In addition, key studies of evolution in *Drosophila* have shown that novelty arises more readily from the recruitment of existing elements into new regulatory networks than from the development of completely new components [3-6]. *Drosophila* is therefore leading the fields of regulatory evolution and speciation[3-11].

It has been argued that reconstructing a system is the ultimate way of understanding it[1,2]. In order to further comprehend the origin of new species,

and to explore possible applications in modern biotechnology, I engineered reproductive isolation between populations of *Drosophila melanogaster* by generating a synthetic species boundary. The generation of a synthetic species may not only help to define the minimal set of regulatory elements required for reproduction barriers but may also partly demystify the process of evolution and speciation by serving as a tool to demonstrate the principles of evolutionary biology[6,7,11].

Previous artificial speciation experiments produced "incipient species" that were not fully isolated or whose speciation genes were unknown[12-15]: Reproductive isolation has been brought about in plants for many decades through polyploidization, which creates individuals that in crosses to parents give rise to sterile progeny[13]. For the animal kingdom, the use of compound chromosomes brings about perfect reproductive isolation that is unbreakable in *Drosophila*, though at the cost of 50% progeny death[14]. Also a new species of lizard (albeit a parthenogenetic one) was recently generated[15].

Unlike previous artificial speciation events, the synthetic species boundary described here is a genetic circuit based on the combination of 5 well known preexisting elements (Fig. 1a) leading to reproductive isolation.

The first element consist of null mutations in the *glass (gl)* gene[19,20]. The *glass* product is a transcription factor of 604 amino acids with five zinc-fingers. Mutations in *gl* specifically abolish photoreceptor cells resulting in blind, but viable flies[19,20]. The gl^{60J} allele is a spontaneous mutant caused by the insertion

of 30kb of unknown DNA into the *gl* locus and it is believed to be a null allele[19,20]. Other alleles (*gl*^{3} and *gl*^{BS1}) have also been used for this study.

The second element is formed by the Glass Multimer Reporter (GMR)[20,21], a heterologous promoter construct containing five tandem copies of a 27-bp *glass*-binding site normally present in the regulatory region of *ninaE*, the major *rhodopsin* gene in *Drosophila*. The GMR promoter can therefore drive *glass*-dependent expression in the photoreceptor cells of *Drosophila* eyes.

The yeast protein GAL4 as a third building block can activate transcription in *Drosophila* from promoters that bear GAL4 binding sites[22,23]. In addition, the GMR sequence has been previously subcloned in front of *gal4*, thus driving Gal4 expression under the control of Glass (*GMR-gal4*)[21].

Fourth, a tandem array of five GAL4 binding sites (5xUAS, for Upstream Activation Sequence) is employed where GAL4 binds with high affinity to induce the transcription of a downstream located gene.

The fifth element is a *ras*^{v12} allele, a mutant form of the *Drosophila ras* gene[24,25]. Conversion of the glycine residue at position 12 to valine constitutively activates the Ras protein. *ras*^{v12} has been previously subcloned behind GAL4 binding sites (*UAS-ras*^{v12}), which permits activation only within cells where GAL4 is expressed[24].

Regulatory evolution acts by using available preexisting genetic elements to generate novelty[3-6]. Likewise, the synthetic genotype created here is achieved by implementing known elements, however their selection and specific

arrangement establishes a previously unknown synthetic species barrier (Fig.1b,c,d). The inherent logic of the design relies on a "killing module" (Fig.1b) and a regulator to switch it ON and OFF (Fig.1c):

The killing module is formed by *GMR-gal4* and *UAS-ras^{v12}* whose activation is controlled by the presence or absence of the gene *glass*. When *glass* gene function is unperturbed, transcription of *UAS-ras^{v12}* driven by *GMR-gal4* consistently kills 100% of the flies at any temperature from 17ºC until 29ºC (606/606 lethality at 17ºC, 558/558 lethality at 23ºC, 330/330 at 25ºC, 110/110 at 29ºC, Table1). More than 20 other *UAS* transgenes were tested, including *UAS-caudal*[26], *UAS-flower^{LoseA}* [27] or *UAS-eiger*[27], but *UAS-ras^{v12}* was the only one that resulted in 100% lethality when driven by the presence of *glass* and the *GMR-gal4* transgene at all temperatures (from 17C to 29C) (Table1).

In the synthetic genotype *GMR-gal4/GMR-gal4; UAS-ras^{v12}, gl^{60J}/UAS-ras^{v12}, gl^{60J}* (a *glass^{60J}* mutant background, where no Glass protein is present) Ras^{v12} cannot be produced (OFF state, Fig.1d). Surprisingly however, in addition to the small eye phenotype (Fig.2a,b), those flies showed a different wing morphology, with lateral extra veins (Fig2d, compare with the wt wing pattern shown in 2c). Other alleles (*gl^{3}* and *gl^{BS1}*) were also tested and yielded the same phenotype. Most likely the heat shock promoter (*hsp70*) of the *GMR-gal4* construct[21] is leaky, leading to very low activation of *UAS-ras^{v12}* and consequently to the phenotype[28].

When hybrids between *Drosophila melanogaster* and the synthetic genotype are produced, the "killing module" *GMR-gal4; UAS-ras^{v12}* is triggered

by the presence of the *glass* gene (Fig.1d, Table 1, Fig.3a). This genetic network, while still allowing normal reproduction among flies with the synthetic genotype, completely isolates *GMR-gal4/GMR-gal4; UAS-ras^{v12}, gl^{60J}/UAS-ras^{v12}, gl^{60J}* flies from normal *D. melanogaster* due to hybrid early pupal lethality (Fig.3a, Table1). Unlike with the other known and naturally occurring speciation mutations[8], the sex of the parents did not influence the lethality of the hybrids in this case (Table1, Fig3a). Experiments were performed at 17ºC because flies of the synthetic genotype grew better and because due to the temperature sensitiveness of the Gal4 it is likely to be the temperature at which the killing module may be less effective. Despite this, the killing module was 100% effective even at 17ºC (Table 1, Fig3a). In the initial population mutations in *yellow (y^{1})*, which results in mild pigmentation, existed as a polymorphism in some individuals.

It is often difficult to delineate "species boundaries" since they may carry identical mutations and are related to one another through common ancestors. However, most biologists agree on a very stringent definition for species, the Ernst Mayr's Biological Species Concept, according to which species consist of populations of organisms that can reproduce with one another, but are reproductively isolated from other such groups[7] (Fig.3b). This definition leads to a focus on the barriers to reproduction between species[8-14]. Such barriers represented one of the main problems for Darwin who wrote: “How can we account for species, when crossed, being sterile (...), whereas, when varieties are crossed, their fertility is unimpaired?”[11]. Because the postzygotically isolated population generated here conforms to Mayr´s and Darwin´s definition of species[8-14], it will subsequently be called *Drosophila synthetica*. Once a

reproduction barrier is formed, traits can be fixed in one of the populations, allowing further divergence[7,11].

To further prove that the synthetic genetic network allowed zero gene flow with *D. melanogaster,* co-cultures of both populations were performed for several generations (using *D.melanogaster white (w)* mutants with white eyes) and not a single hybrid was recovered (Fig. 3c, Table1). Hybrids would have been easily recognizable by normally sized red eyes, because they would carry a normal copy of *gl* and two *w+* copies from the transgenes (Table1), but *D. synthetica* behaved like a stable species, did not reproduce and maintained its characteristic eyes (Fig.3c).

Assembling synthetic species boundaries can have practical applications. For example, the use of recombinant DNA technology to alter organisms for a specific purpose has raised controversy[18] and is a growing problem due to the increasing number of transgenic organisms approved by regulatory agencies[16-18]. A new framework where safety mechanisms are genetically designed along with desired modification could help to gain public support for a technology with the potential to satisfy future medical and nutritional needs[16-18]. *D. synthetica* is the first transgenic organism that cannot reproduce with the original *wildtype* population. I therefore propose that synthetic species barriers may serve to compartmentalize dangers and protect natural species from interbreeding with emergent transgenic forms, therefore preserving natural biodiversity (Table1).

Moreover, once a genetic network is identified, as it is the case for the "*ras-glass*" synthetic boundary described here, opening or closing of the barrier

can be controlled at will. In case the interbreeding of populations appears beneficial, targeted strategies can be implemented to reverse hybridization barriers. To test this experimentally, the GAL4-inhibitor GAL80 was expressed from a *tubulin* promoter (*tub-gal80*) in *D. melanogaster* in order to remove hybrid lethality and traverse the species barrier. Males of *D. synthetica* hybridized successfully with *tub-gal80 D. melanogaster* females and produced viable hybrids (Table1), as predicted because Gal80 can block the "killing module".

The ability to open and close speciation gates when desired reflects one of the goals of synthetic biology –to build components that can be reliably and predictably manipulated[1,2]–, and preserves flexibility while gaining control over the spread of genetically modified organisms.

The generation of *D. synthetica* conforms to the Dobzhansky-Muller theoretical model for postzygotic incompatibilities during naturally occurring speciation[7,8], according to which an ancestral population splits into two independent populations that then accumulate mutations (Fig.3b). Subsequent genetic interactions between those mutations cause hybrid incompatibilities. In particular, it conforms to a derived-ancestral incompatibility (Fig.3b), in which all substitutions occur in the derived population. One of the predictions of classical evolutionary theory is that "missing links" (organisms that connect two species) must exist as part of the gradual divergence process[11]. Because we fully know the mutations forming a reproduction barrier between *melanogaster* and *synthetica*, it is feasible to dissect the process and move backwards, showing how populations of intermediate mutants can indeed interbreed with populations

at either side of the evolutionary path towards postzygotic isolation (i.e., *glass* mutants can hybridize with both species (Table1) and hence constitute a "missing link" connecting *melanogaster* with *synthetica*) (Fig.3).

It is likely that modifications in transcription factors (e.g. *glass* mutation) and appearance of cryptic enhancers upstream of potentially lethal gene products (e.g. GMR-mediated activation of ras^{v12}), or even in front of genes whose eventual repression will cause lethality, can constitute a normal Dobzhansky-Muller mechanism for speciation (Fig.3d). The appearance of those cryptic enhancers could be driven by the accumulation of point mutations in regulatory regions (Fig.3d), in a manner similar to what has been described recently[30], but those enhancers will only be recognised by the ancestral transcription factor which is now missing (or modified) in the derived population (Fig.3d). When hybridization between the derived and ancestral populations occurs, the genes with cryptic enhancers will be activated by the ancestral transcription factor, causing hybrid lethality and reproductive isolation (Figs.1,2, Table1). This could constitute a general mechanism through which regulatory evolution creates species boundaries (Fig.3d) and may help to define concrete target genes mediating speciation. Against this, it could be argued that, because the flies are blind and only survive at lower temperatures, they have fitness deficits and the changes could not be arrived at in concert; nor could the "fitness valley" be traversed in the wild. However, blindness is a common adaptation in caves, where temperatures are also low, strongly counteracting this argument[7,11].

Similarly, one could ponder whether the strain is fit enough to be used for field studies where it would need to compete with wild type flies. However, this is hardly a criticism regarding its practical applications, because the transgenic strain should rather be isolated and not compete with the wild populations.

Another criticism could be that this barrier is not irreversible since it can be reversed quite simply if a spontaneous mutation was to arise in any of the components. If we think in terms of engineering or synthetic biology, having fail safe mechanisms in a machine makes it safer, despite they can fail themselves. The solution is therefore just to add more fail safe mechanisms. Identically, adding more synthetic speciation barriers, with the same logic, will make it also safer and less reversible. Other transcription factors and enhancers could be easily used to create those extra barriers, because the concept goes beyond any particular element. Importantly, modification of the binding properties, instead of complete elimination of the transcription factor, could also be implemented, reducing the constraint of not finding enough non-essential transcription factors to build several barriers.

In summary, this study described the first transgenic animal that cannot hybridize with the original wild type population but remains fertile when crossed with other transgenic animals. This provides proof of principle for the transition from “transgenic animals” to “synthetic species”, and should spur the debate for its use as a failsafe mechanism in biotechnology.

Materials and Method

High definition and depth of field photographs were obtained with a Keyence VHX-600 microscope. Flies were frozen at -20°C overnight before imaging. For SEM, adults were fixed in 2.5% glutaraldehyde in PBS overnight at 4°C, post-fixed in 1% osmium for 2 h at 4°C, washed, dehydrated in ethanol and with Hexamethyldisilazane until evaporation of the solvent. Samples were coated with 30 nm of gold and observed with a 440 Leica microscope under 20 kV tension.

The fly stocks used were obtained from the Bloomington Stock Center except where indicated. The following stocks were used: *GMR-gal4, UAS-ras^{v12}, glass^{60J}, UAS-Dpp, UAS-wg-HA*, *UAS-egr, UAS-brk (*G.Campbell*), UAS-hep^{CA}, UAS-fwe^{Lose-A}* and *UAS-fwe^{Lose-B}*, *UAS-hid* (H. Steller), *tub-GAL80.*

For the balancing of the different transgenes the following stocks were used:

ywhs-FLP;If/CyO; MKRS/TM6b

w^{1118}; Pas^{SC1} gl^{3}/TM6B, gl^{BS1} Tb^{1}

w^{1118}; If/CyO; MKRS/TM6B, gl^{BS1} Tb^{1}

C(1)DX,y^{1},f^{1},hs-hid

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Figure legends

Figure 1: Design of a genetic circuit with selected components that form a synthetic species barrier.

(A) The 5 genetic elements used: transcription factor *glass*, enhancer *GMR*, transcription factor *gal4*, enhancer *UAS* and a constitutively activated form of *ras*. (B,C) Arrangement of the genetic elements in two modules, a killing module (B) composed by two independent transgenes, *GMR-gal4* and *UAS-ras*^{v12},and a switch that depending on the presence or absence of the transcription factor *glass* can switch the killing module ON and OFF (C). (D) In the absence of Glass, activation of the killing module is not possible and the flies survive. However, in the presence of Glass, expression of the constitutively active form of *ras* kills the animal.

Figure 2: Morphological traits of *Drosophila synthetica*.

(A-B) Scanning electronic microscopy (SEM) images of *Drosophila synthetica* flies. Eye is small due to lack of *glass*. (C-D) Wings of *Drosophila synthetica* show extraveins in the lateral regions of the wing (D) compared to the *Drosophila melanogaster* wing (C).

Figure 3: Creation of species boundaries by regulatory evolution. (A) Hybrids between *melanogaster* and *synthetica* arrest in pupae and do not develop further, even at 17C. The sex of the parents did not affect the outcome. Pupae shown in the pictures are more than one month old. (B) Scheme of a

classical Dobzhansky-Muller mechanism for speciation, where all mutations occur in one of the populations ("derived"), and the hybrids between the "ancestral" (aabb) and "derived" (AABB) populations are lethal. (C) High definition and depth of field images of *Drosophila synthetica* after several generations of coexistence with *D. melanogaster*. Image obtained with a Keyence VHX-600 microscope. Eyes are pale in addition to small. A *D.melanogaster* eye is shown for comparison in the upper right corner. (D) General model for the creation of species boundaries based on the modification of transcription factors and the subsequent appearance of cryptic enhancers. This could be a mechanism to create synthetic species and prevent hybridization of transgenic animals with natural populations. The case of *Drosophila synthetica* is shown. Years correspond to the first appearance of the mutation or transgene in a *Drosophila* laboratory.

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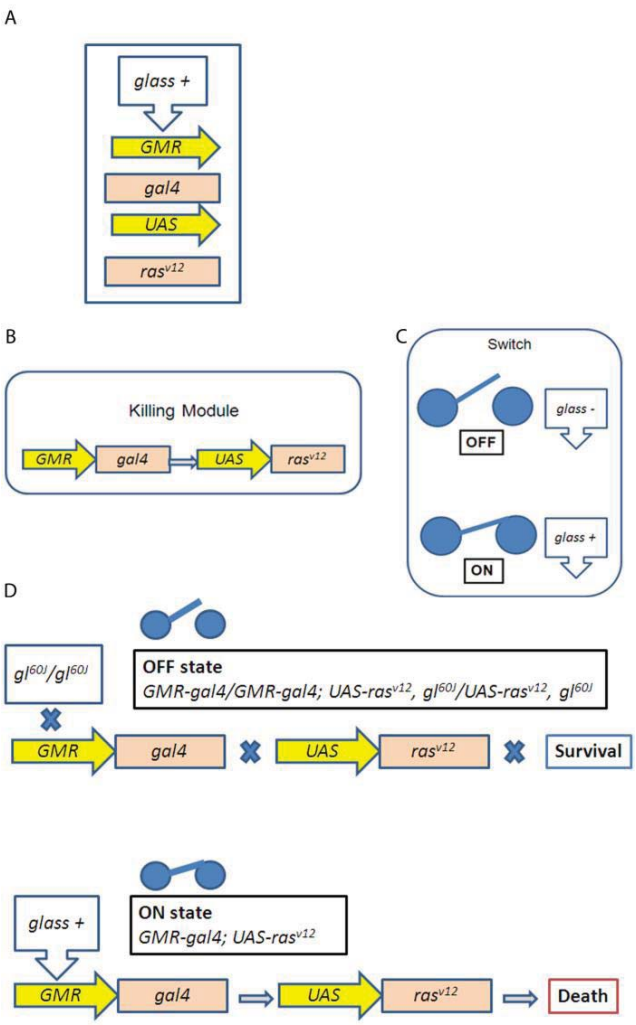
A
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glass +
D
gl^60J/gl^60J
OFF state
GMR-gal4/GMR-gal4; UAS-ras^v12, gl^60J/UAS-ras^v12, gl^60J
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gal4
UAS
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Survival
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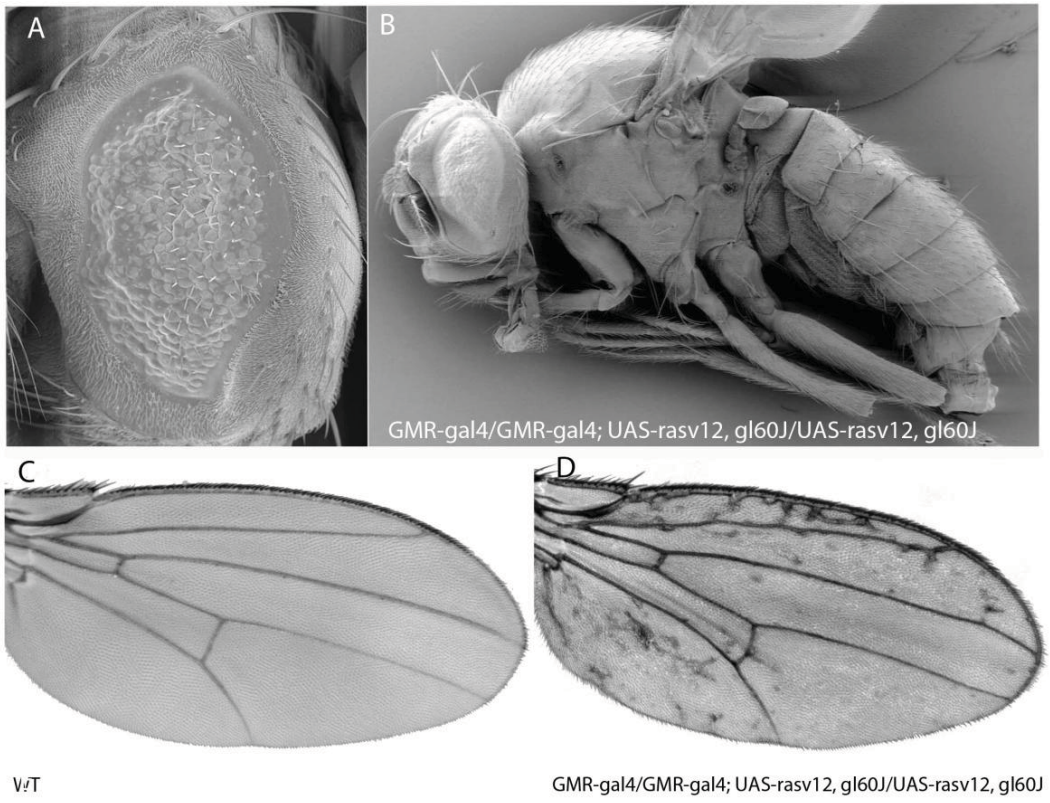
GMR-gal4; UAS-ras^v12
GMR
gal4
UAS
ras^v12
Death

Figure 1

Figure 2.


A
B
GMR-gal4/GMR-gal4; UAS-rasv12, gl60J/UAS-rasv12, gl60J
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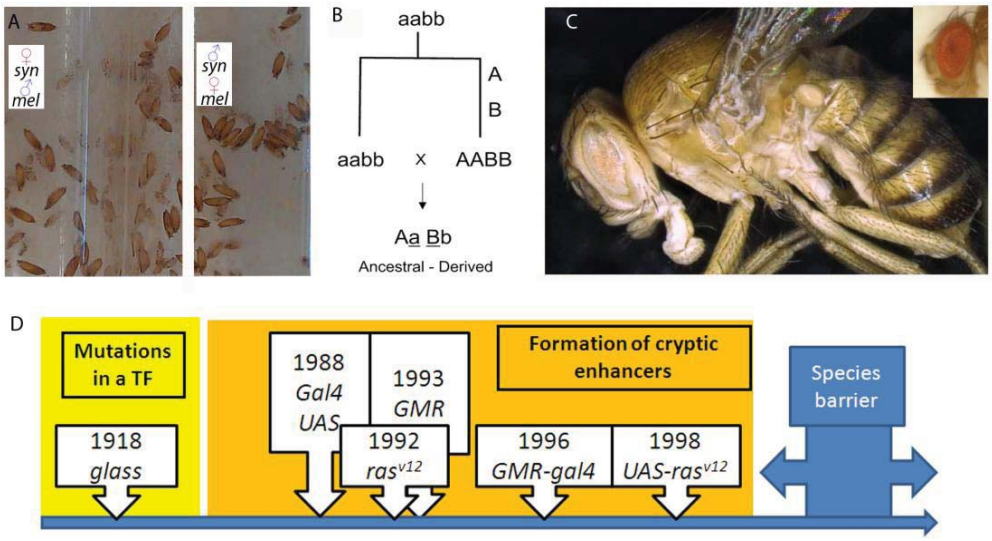
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Aa Bb
Ancestral - Derived
C
D
Mutations in a TF
1918 glass
1988 Gal4 UAS
1993 GMR
1992 ras^v12
Formation of cryptic enhancers
1996 GMR-gal4
1998 UAS-ras^v12
Species barrier

Figure 3

Table 1.

Parental genotypes	Number of F1 adult progeny	Number of dead pupae
♂ ***GMR-gal4 / GMR-gal4*** ***x*** ♀ ***UAS-ras^{v12} / UAS-ras^{v12}***	0 (no survivors at any temperature from 17ºC to 29ºC)	606/606 lethality at 17ºC 558/558 lethality at 23ºC 330/330 lethality at 25ºC 110/110 lethality at 29ºC
♂ ***GMR-gal4/GMR-gal4; UAS-ras^{v12}, gl^{60J}/UAS-ras^{v12}, gl^{60J}*** ***x*** ♀ ***GMR-gal4/GMR-gal4; UAS-ras^{v12}, gl^{60J}/UAS-ras^{v12}, gl^{60J}***	>1000 at 17ºC >1000 at 25ºC	0 at 17ºC 20/100 at 25ºC
♂ ***GMR-gal4/GMR-gal4; UAS-ras^{v12}, gl^{60J}/UAS-ras^{v12}, gl^{60J}*** ***x*** ♀ ***Oregon R***	0 (no survivors)	293/293 lethality at 17ºC
♂ ***Oregon R*** ***x*** ♀ ***GMR-gal4/GMR-gal4; UAS-ras^{v12}, gl^{60J}/UAS-ras^{v12}, gl^{60J}***	0 (no survivors)	50/50 lethality at 17ºC
♂&♀ ***w; GMR-gal4/GMR-gal4; UAS-ras^{v12}, gl^{60J}/UAS-ras^{v12}, gl^{60J} (genotype 1)*** ***x*** ♂&♀ ***w/w (genotype 2)***	>1000 of genotype 1 >1000 of genotype 2 0 hybrids (red normally sized eyes)	>1000 at 17ºC
♂ ***GMR-gal4/GMR-gal4; UAS-ras^{v12}, gl^{60J}/UAS-ras^{v12}, gl^{60J}*** ***x*** ♀ ***tub-gal80/ tub-gal80***	71 at 25ºC	0 at 25ºC
♂ ***GMR-gal4/GMR-gal4; UAS-ras^{v12}, gl^{60J}/UAS-ras^{v12}, gl^{60J}*** ***x*** ♀ ***gl^{60J}/gl^{60J}***	87 at 25ºC	0 at 25ºC